SolNanoTox

How the solubility of nanoparticles impacts on their behaviour and their potential toxicity?

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anses
French agency for food, environmental and occupational health & safety

Program between France and Germany

Up to 42 months; from March 2014 up to August 2017

Behaviour, toxicity and mechanism of action of NMs through oral exposure not well established

Due to the large panel of NMs, is solubility a key factor in hazard assessment?

Study interactions and toxic effects
with 2 types of NMs,
one soluble and one insoluble

Choice of MNMs

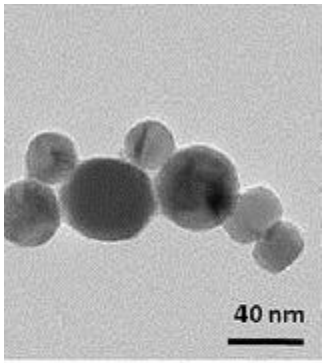
2 types of NMs but of similar size

commonly used in food, or expected to migrate through food processing or materials in contact

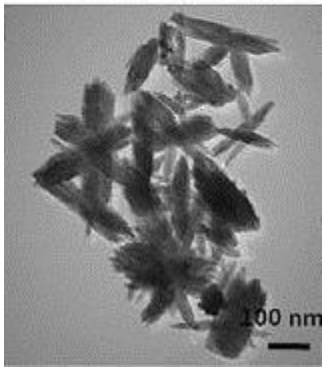
Will or may be ingested by consumers

Choice of MNM

TiO2



Anatase



Rutile

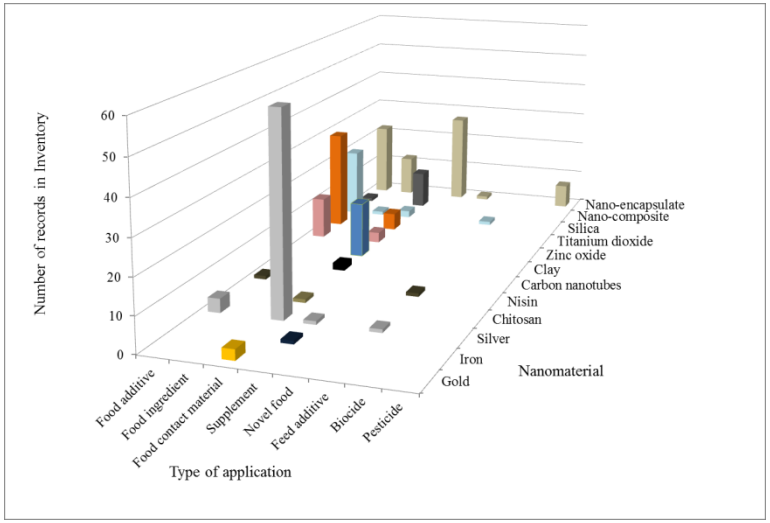
Anatase would produce more oxidative stress and suggested to be more toxic (Xue et al., 2010; Petkovic et al, 2011, review of Wang and Fan, 2014)

But other studies showed that rutile induces higher toxicity (Numano et al , 2014 ; Sund et al, 2014)

Only few data published on the toxicity (especially *in vivo*) of the rutile forms

Choice of MNMs

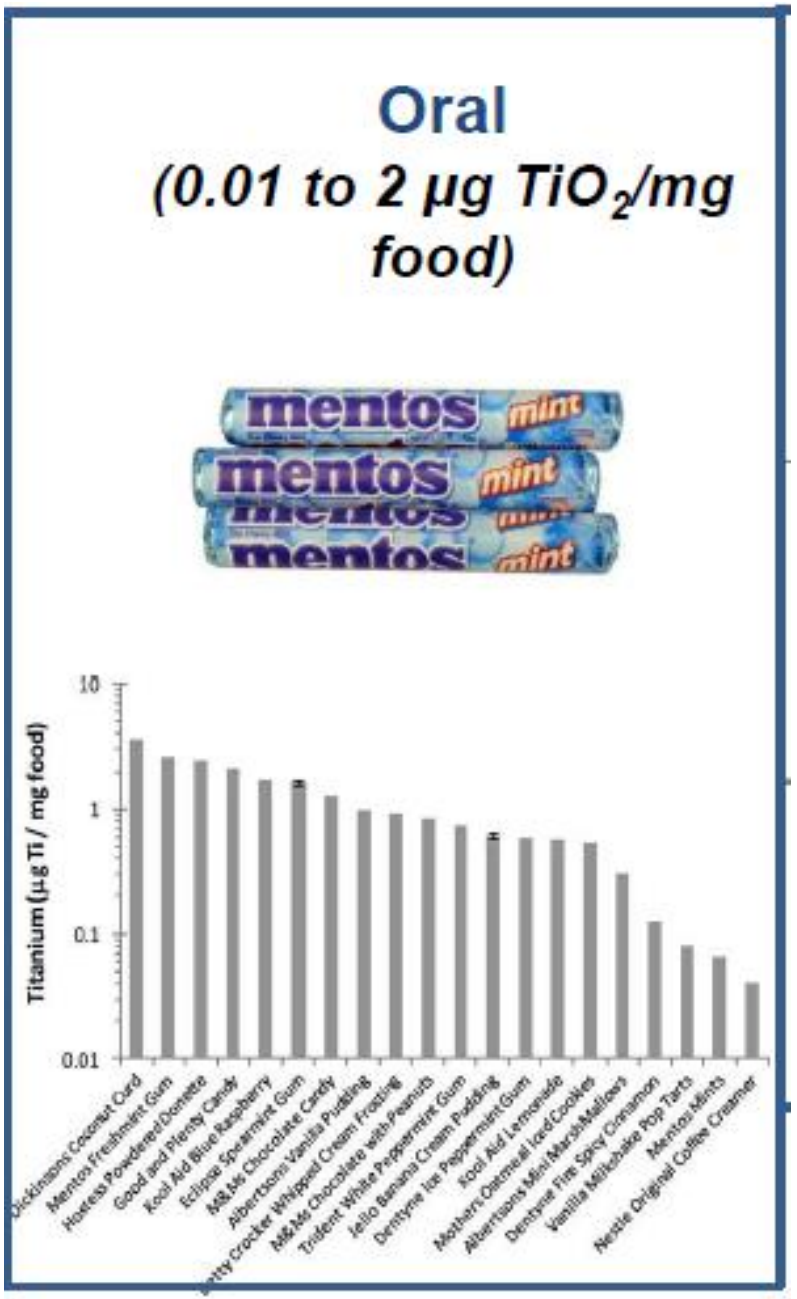
TiO2 uses in food products



NPs applications in the agricultural, feed and food sector

EFSA report (2014)

Chen *et al. 2012, Weir et al., 2012, Powell et al. 2000*



Choice of MNMs

TiO2: hazardous?

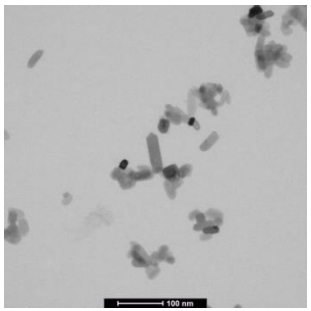
International Agency for Research on Cancer:
"**possibly carcinogenic to humans" (2B)**

Genotoxicity data *in vitro* and *in vivo*: contradictory results
Probably oxidative stress induction of DNA damage but not excluded that an direct effect may also occur

Biodistribution: some accumulation in liver (Shukla et al 2013; Geraets et al 2014)

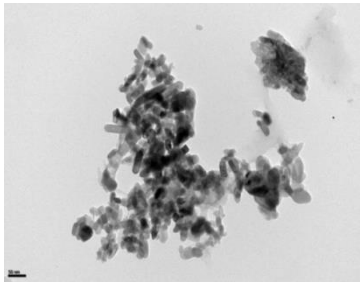
Choice of MNMs

TiO_2 (from JRC)



NM103
(thermal, hydrophobic, coated)

25 nm



NM104
(thermal, hydrophilic)

Choice of MNMs

Aluminium

2 nanosized forms:

Al^0

Oxide: γAl_2O_3

Similar size to TiO_2 around 20 nm

Comparison with the ionic release by $AlCl_3$

Choice of MNMs

Aluminium uses

- highly abundant, mostly in oxides, hydroxides or salts

- **natural component** of food and drinking water
- in **direct contact** with food via packages, foils and kitchen ware
- Aluminium-containing **food additives** in many products (confectionary, spices, cheese, candied cherries, biscuits, medical capsules)

Choice of MNMs

Aluminium toxicity

- considered to cause bone diseases, anemia, cancer and neurodegenerative disorders

- uptake, biochemical effects and health hazards are widely unknown

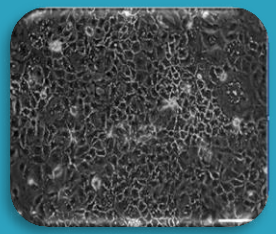
Choice of models

In vitro:

- Cell models of human origin
- Close characteristics to primary cells of the selected tissue
- Good knowledge in the labs
- Representative of the organ of entry (intestine) and the organ of accumulation (liver)

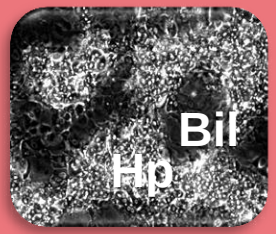
Caco2

Mimicking enterocytes



HepaRG

Mimicking hepatocytes and biliary cells



In vivo:

Small intestine and liver of rats

Key issues for the SolNanoTox project

- **Quantification of internal doses of nanomaterials (NM) to estimate Dose-Response relationships**
- **Influence of the human processes like digestion and intestinal mucus secretion on the kinetics, the dynamic behaviour and the biological effects of MNMs?**
- **Stress responses and pathways associated to MNM exposure in liver and intestine?**
- **How solubility impacts NM behaviour and toxicity**
- ***In vivo* extrapolation from *in vitro* biological effects?**

For achievement

Use of **complementary methods** to:

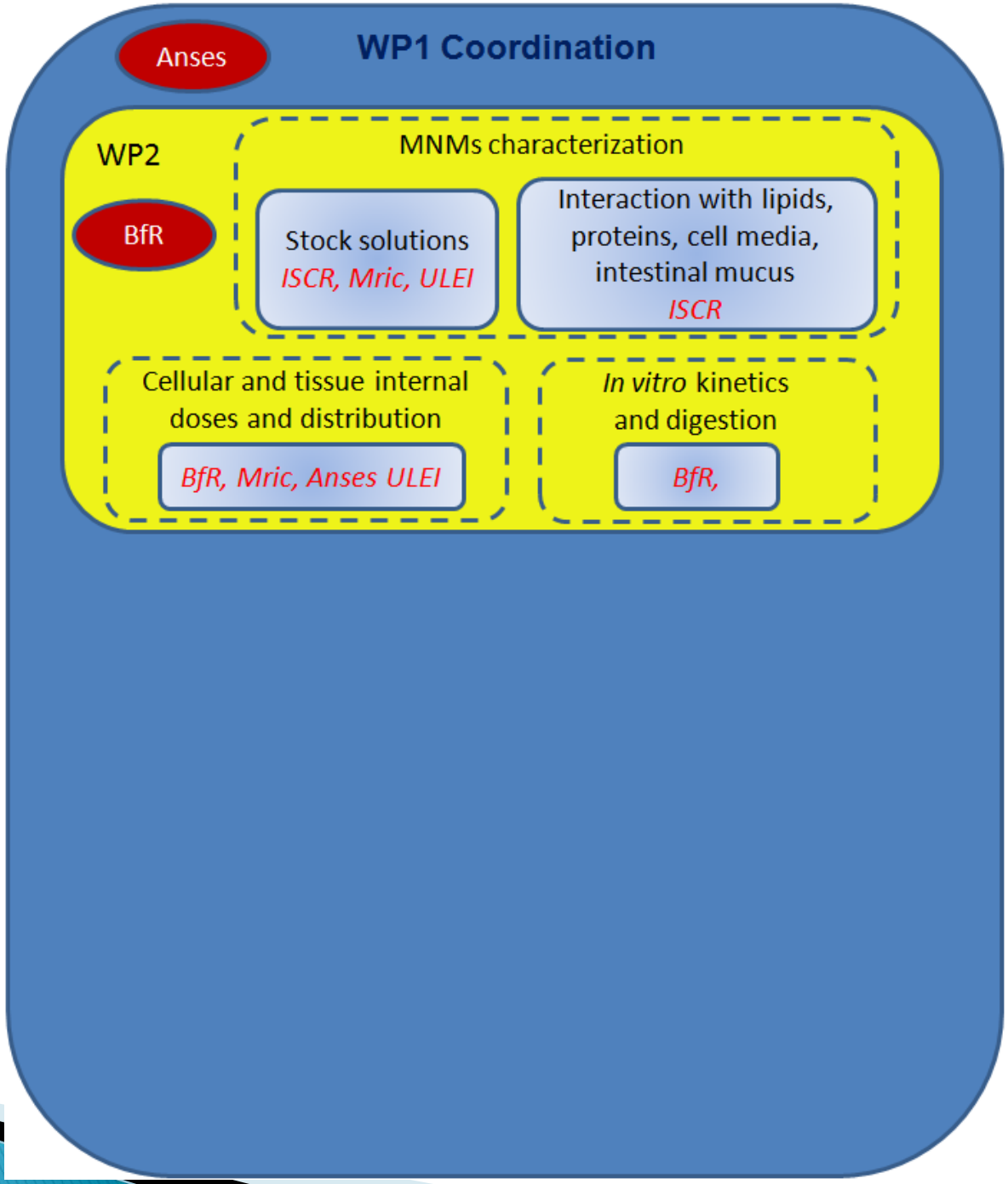
- Measure the physic-chem characteristics of NMs

- Detect and quantify the uptake into cells and tissues

- Determine the biological effects at the molecular, cellular and organ levels with an overview of the cytotoxic and genotoxic responses induced and identification of biomarkers and mechanisms of toxicity

Use of **newly developped and highly performant techniques**

Combination of **integrative *in vitro* and *in vivo* approaches**


Anses
WP1 Coordination
WP2
BfR
MNMs characterization
Stock solutions
ISCR, Mric, ULEI
Interaction with lipids, proteins, cell media, intestinal mucus
ISCR
Cellular and tissue internal doses and distribution
BfR, Mric, Anses ULEI
In vitro kinetics and digestion
BfR,

WP2

Task 2.1 & Task 2.2

Characterization of MNMs in stock solution

Interaction of NMNs with cell media and intestinal mucus

Dispersion protocol of the MNMs:

- NANOGENOTOX protocol (also use in NanoReg)

- in distilled water + 0.05% BSA, sonication during16 min

- solution of 2.56 mg/ml

Various methods (DLS, NTA, XRD, zeta potential, TEM, Tof SIMS,…) for physic-chemical characterization

WP2

Task 2.1 & Task 2.2

Characterization of MNMs in stock solution

Interaction of NMNs with cell media and intestinal mucus

TiO2 In stock solution (dispersion medium), data from the European Joint Action Nanogenotox (2010-2013) and the FP7 project NanoReg (2013-2017)

Al Behaviour and characteristics in stock solution (dispersion medium) to be obtained

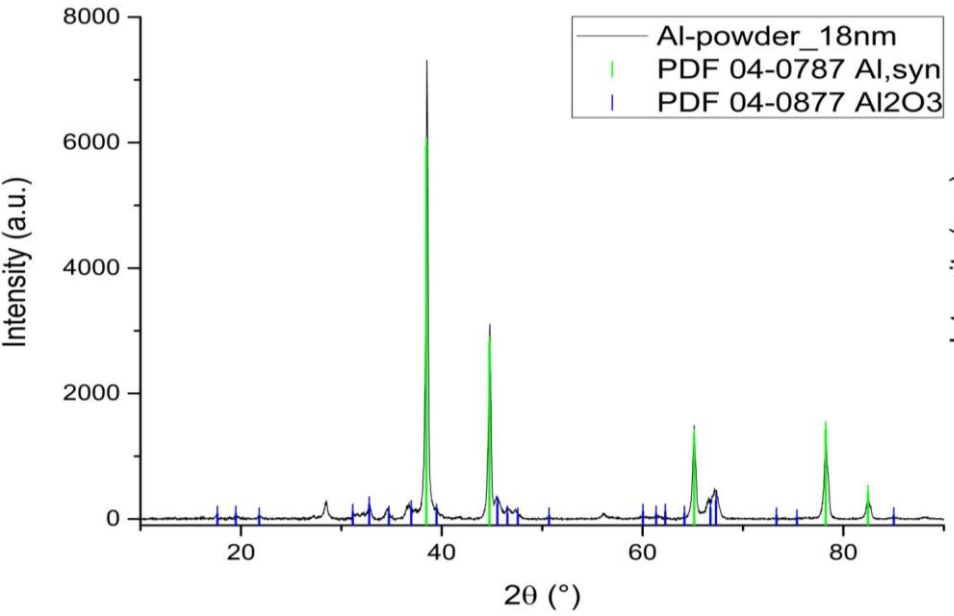
Complete or obtain the data in the cell media used in *in vitro* studies including the stability over time of exposure

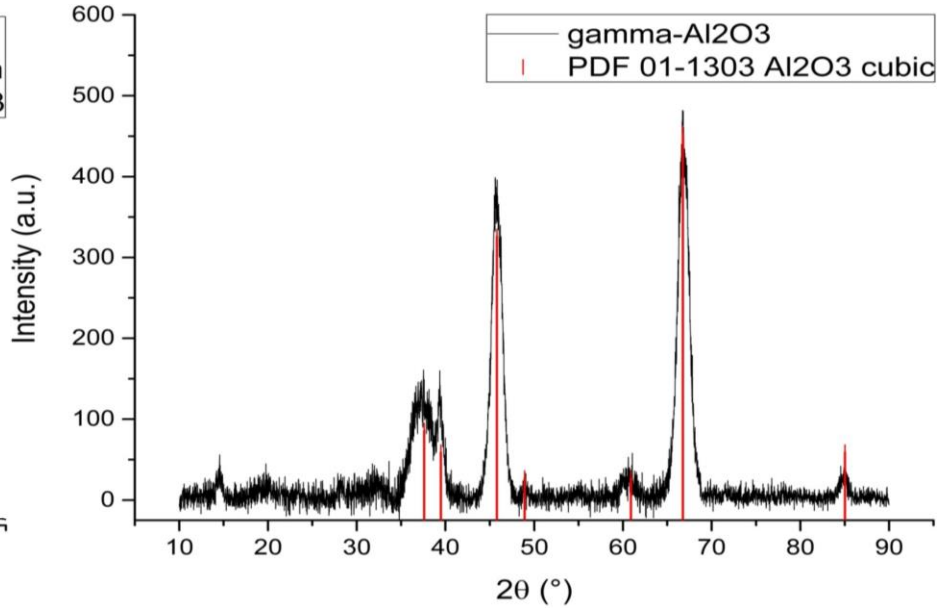
Study the interaction with mucus produced by human intestinal cell models *in vitro* and with purified mucins

WP2

Task 2.1
& Task 2.2

Characterization of Al NMs



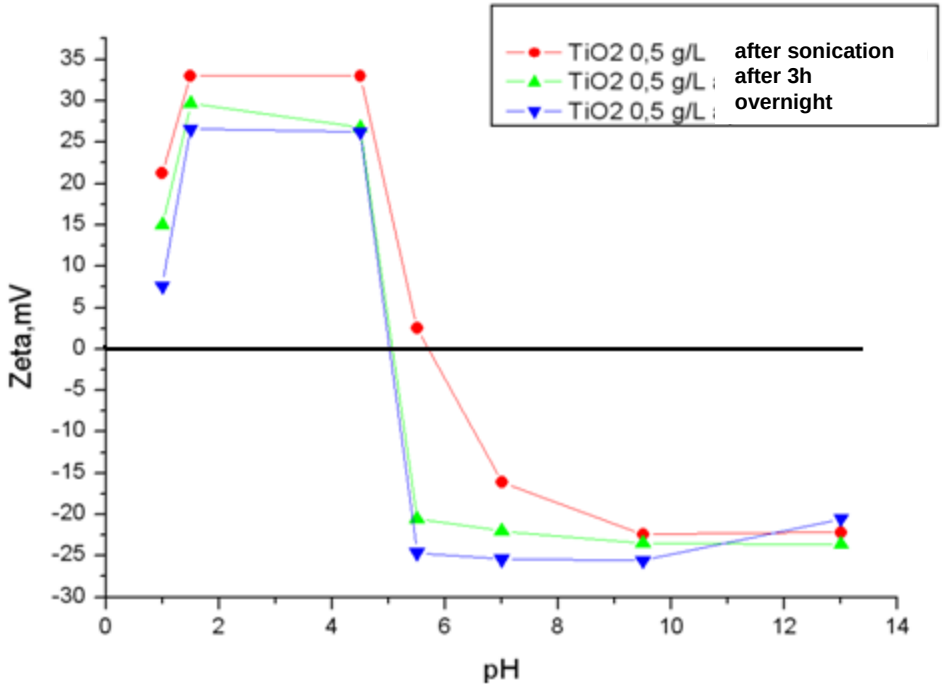


Investigation by X-ray diffraction revealed a thin Al oxide layer at the surface of the metallic Al particles

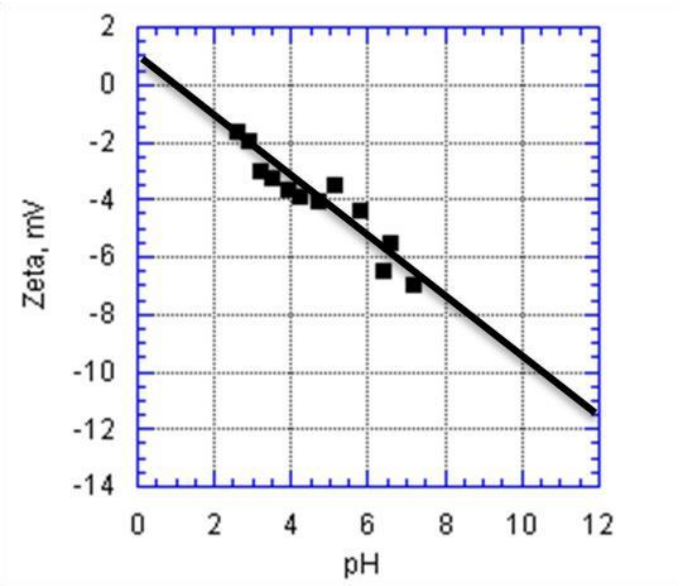
WP2

Task 2.1 & Task 2.2

Interaction of TiO2 with mucins



Zeta potential of TiO2 NM-104 with time CNPs=0.5 g/L



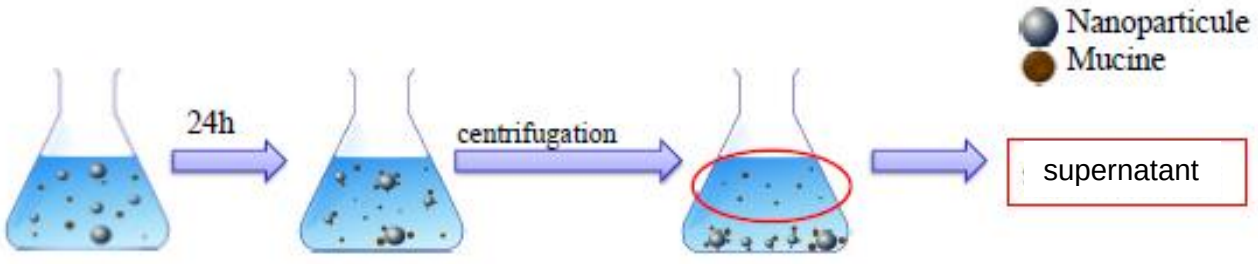
Zeta potential of mucin type III within a pH range (Cmucin = 0.5 g/L)
Mucin from stomach of pig

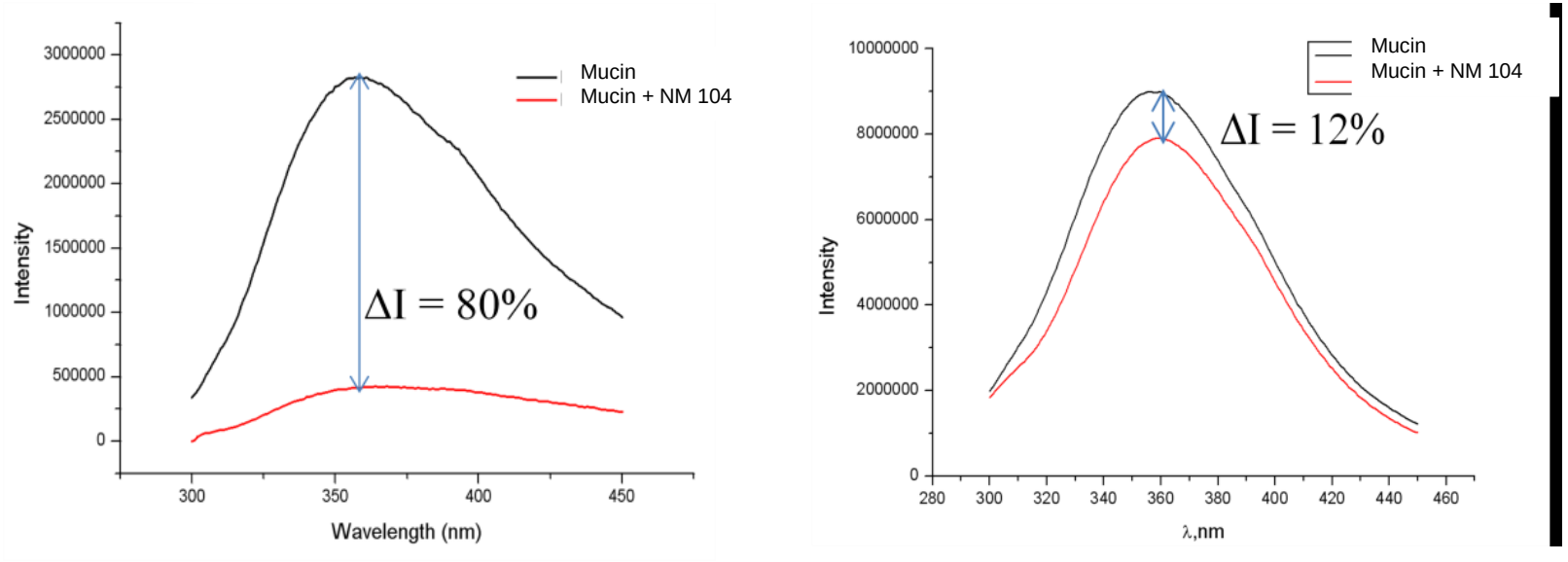
NM104 are positively charged with stomach pH conditions (below 4) and negatively charged in intestine (pH above 6)
Mucin III is negatively charged independently of pH value

WP2

Task 2.1
& Task 2.2

Interaction of TiO2 with mucins





Fluorescence spectra of supernatants from mucin alone or in the presence of TiO2 NM-104 at pH 3.5 (left) and pH 8 (right). λex=280nm

At pH=3.5 NM104 positively charged adsorbed on mucin which are negatively charged.

At pH 8 NM104 negatively charged and no interaction with mucin due to repulsion forces.

WP2

Task 2.3 & Task 2.4

Internal exposure *in vivo*: Quantification and characterisation of particle uptake in gut and liver

Internal exposure *in vitro*: Quantification and characterisation of particle uptake in gut and liver cells

Methodology:

-TEM
- IBM
- ToF- SIMS
- SP-ICP-MS
- Raman spectroscopy

Use of complementary approaches

Not only uptake into cells but also distribution inside the cells/ the tissues as well as quantification

WP2

Task 2.3
& Task 2.4

Uptake *in vitro*: First results on TiO2

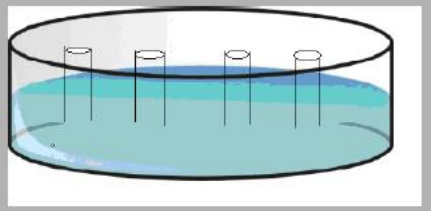
TEM conventionnal approach

Caco 2 cells

Contact 24h
NM 103
NM 104 (256 to 20 µg)

1 rinsing

MET preparation technique

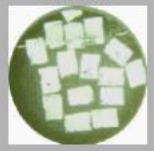


Recovering blocks with cells monolayer

Cutting step

90nm thickness sections

Grid



3mm

TEM analysis 120 Kv,

WP2

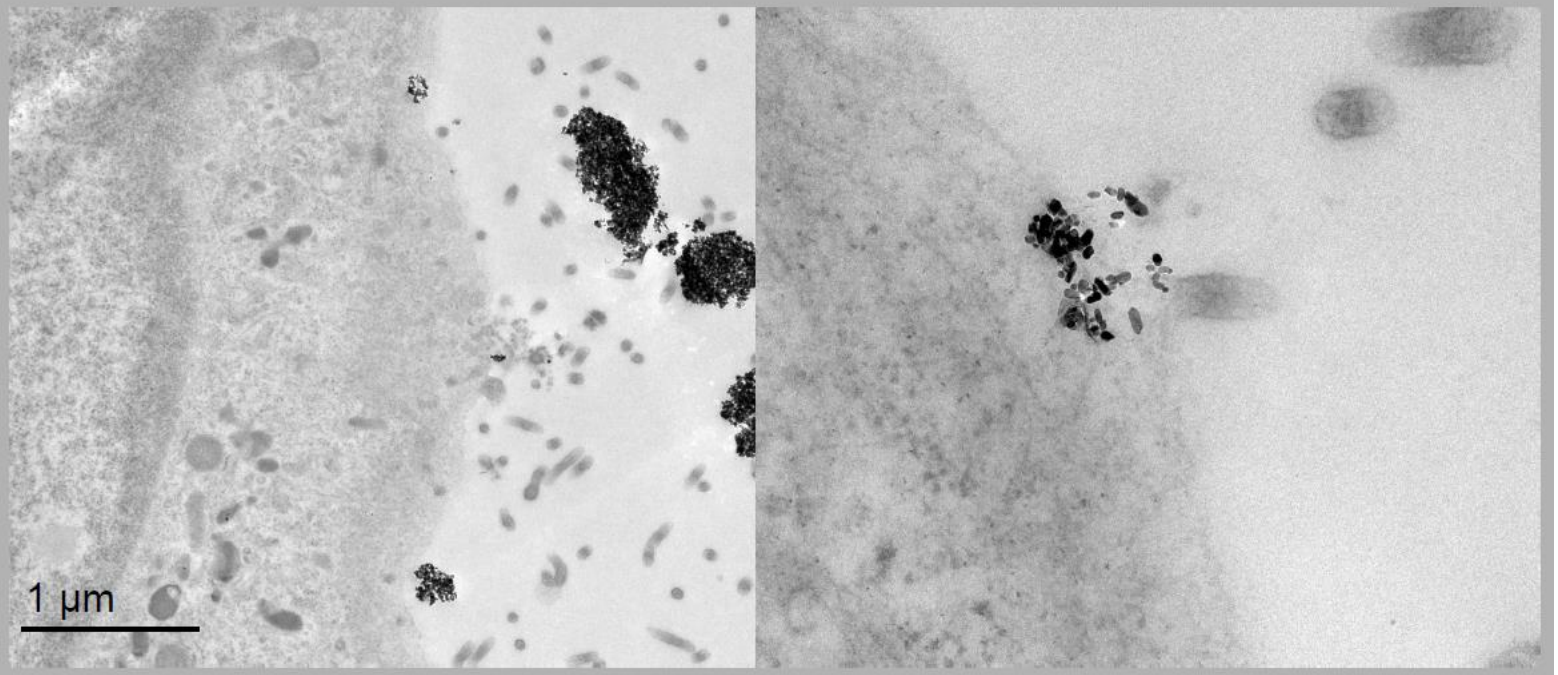
Task 2.3
& Task 2.4

Uptake *in vitro* in Caco2 cells: First results on TiO2

NM entrance into the cells

NM 103-128-15k

NM 103-128-80k



WP2

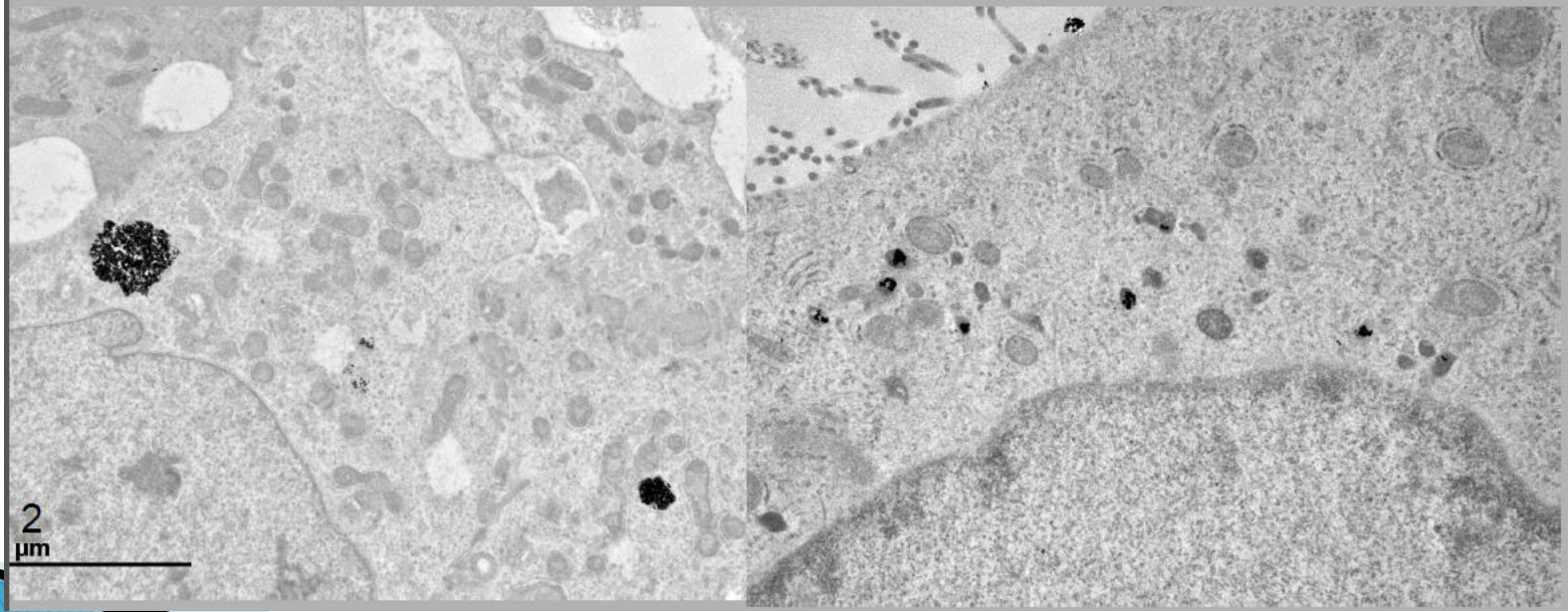
Task 2.3
& Task 2.4

Uptake *in vitro* in Caco2 cells: First results on TiO2

NM inside the cells

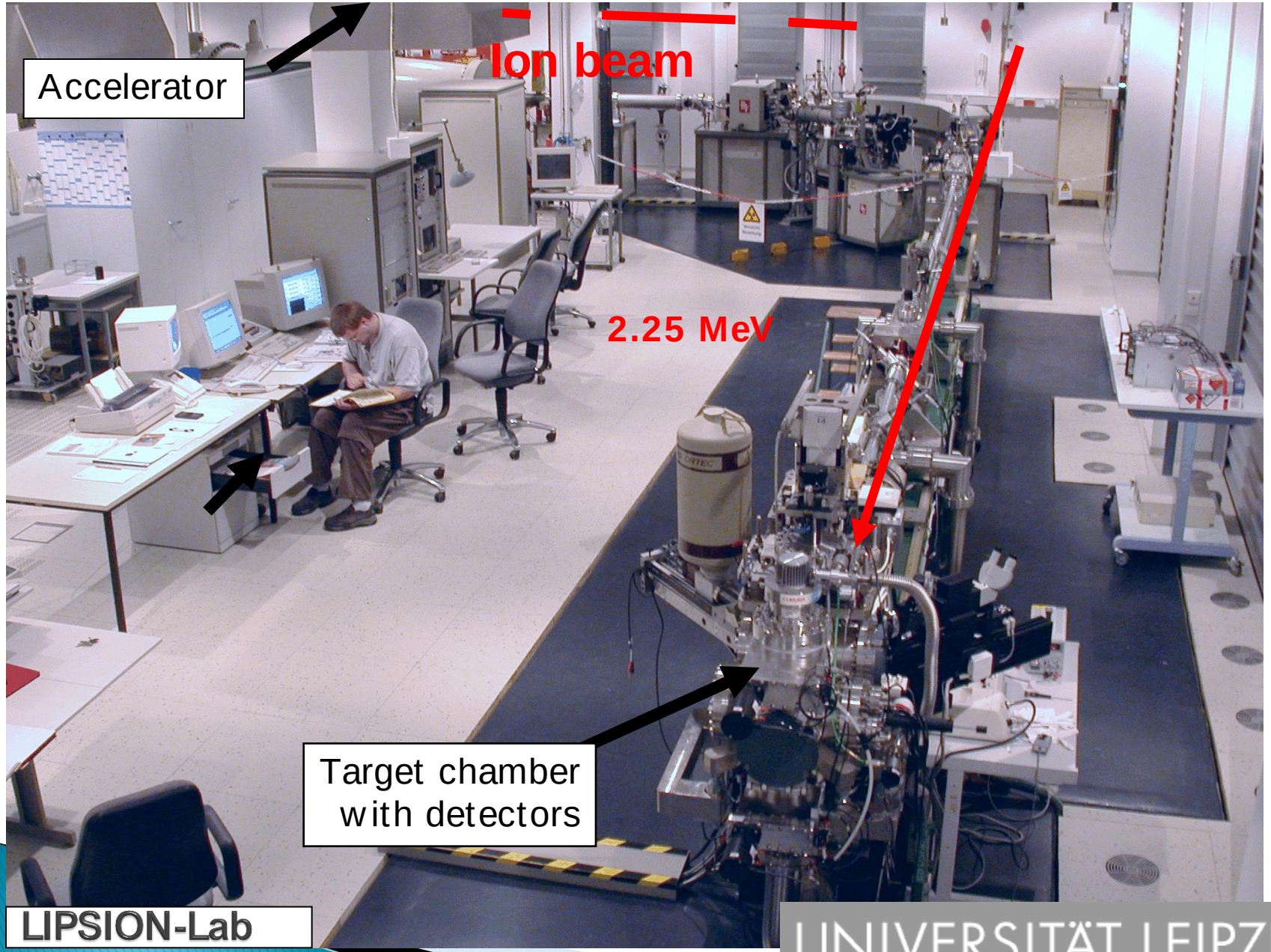
NM 103-128-8k

NM 104-256-20k



Both NM103 and 104 whether in free form or inside vesicles (endosomes?)

The quantification of the NPs uptake *in vitro* and in *vivo* at single cell level by Ion Beam Microscopy



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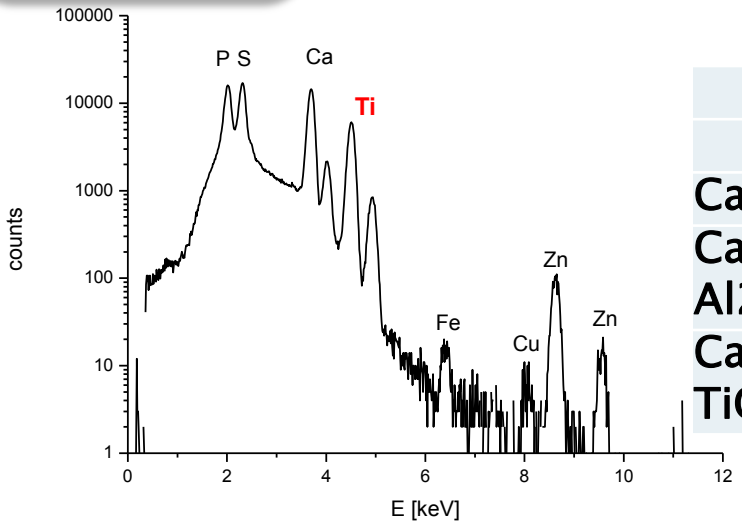
WP2

Task 2.3 & Task 2.4

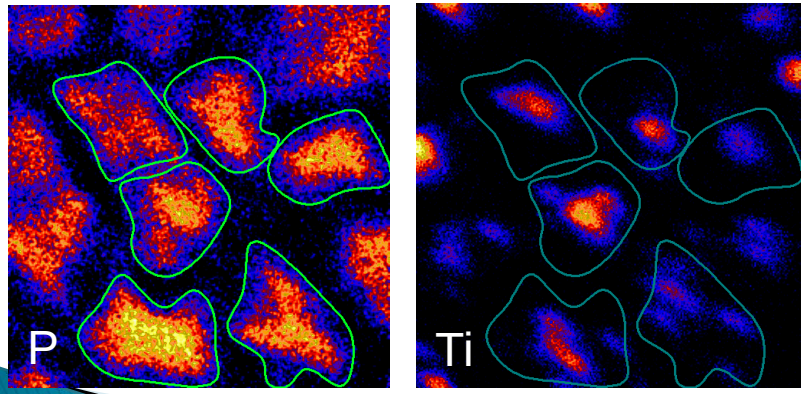
The quantification of NP uptake in single cells (IBM)

PIXE

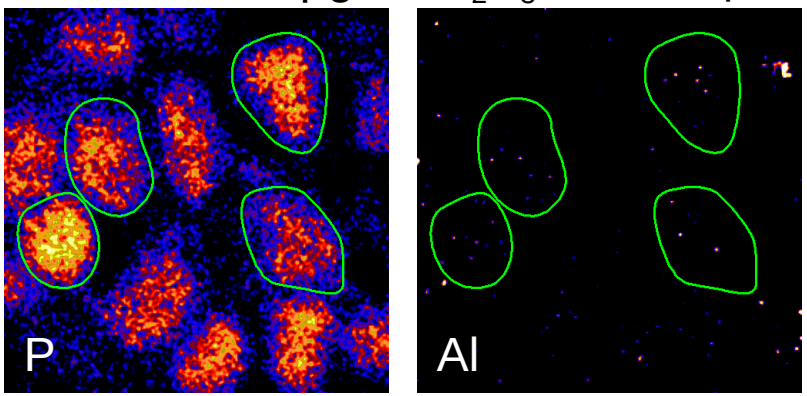


	Elemental Concentration [ppm]		
	P	Ti	Al
Caco-2 Control	13120	0	0
Caco-2 + 50µg/ml Al2O3	9810	0	230
Caco-2 + 50µg/ml TiO2	11500	4790	0

Caco-2 + 50 µg/ml TiO_2 – 24 h exposure



Caco-2 + 50 µg/ml Al_2O_3 – 24 h exposure



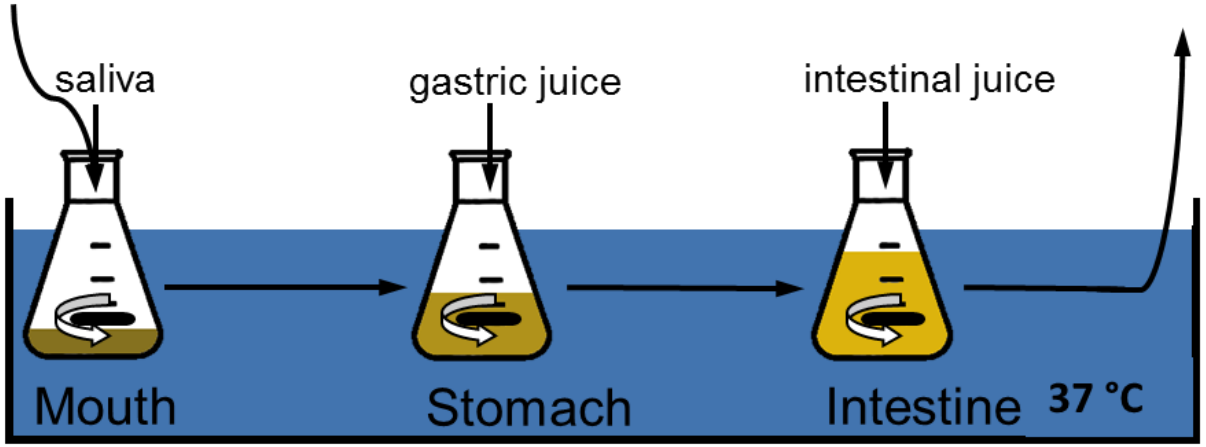
scanfield 50 x 50 µm

WP2
Task 2.5 &
Task 2.6

In Vitro simulation of different digestic models

Impact of the digestion process: *In Vitro* Digestion

- NMs can be *in vitro* digested in **artificial fluids**
- simulate way through **gastrointestinal tract**

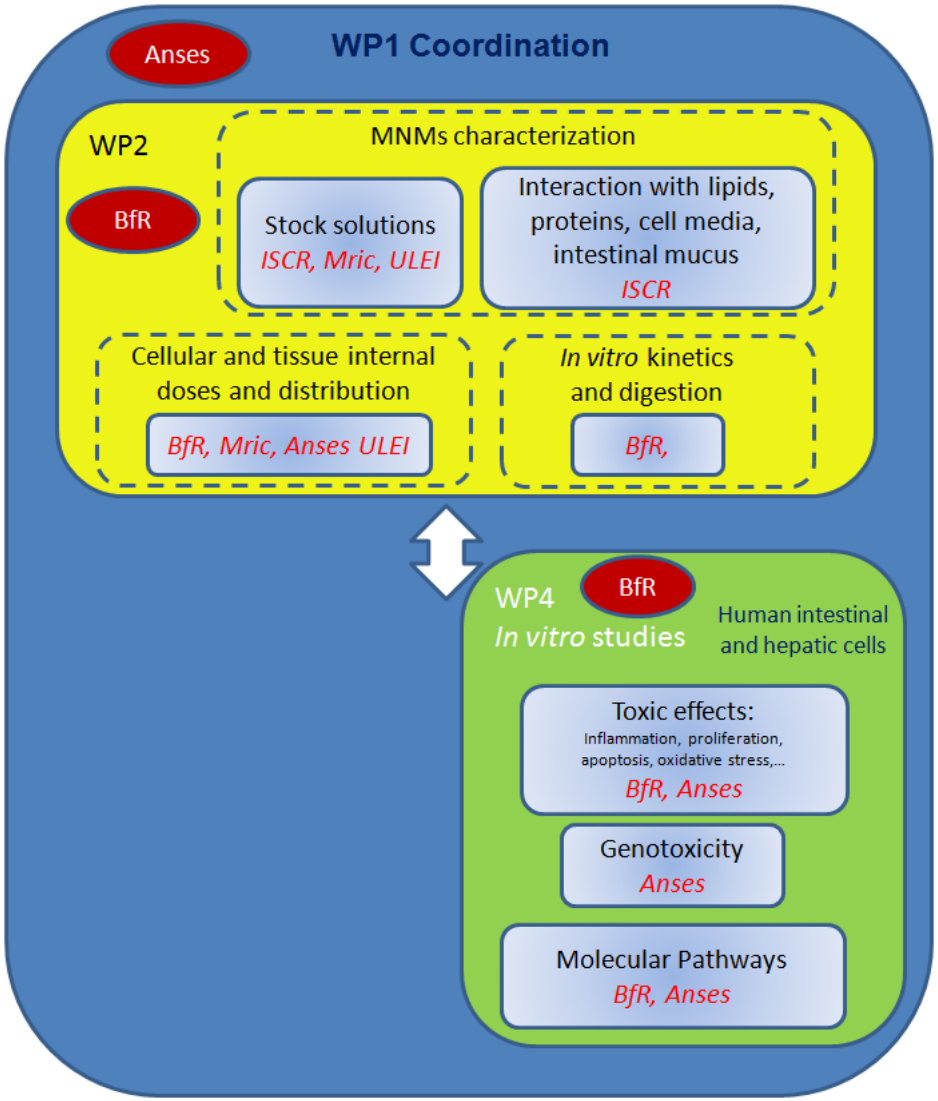


Characterization of digested NMs

Molecular pathways using microarrays

Uptake and transport across *in vitro* intestinal barrier models

Toxicity markers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Cellular and tissue internal doses and distribution
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In vitro kinetics and digestion
BfR,
WP4
In vitro studies
BfR
Human intestinal and hepatic cells
Toxic effects:
Inflammation, proliferation, apoptosis, oxidative stress,...
BfR, Anses
Genotoxicity
Anses
Molecular Pathways
BfR, Anses

WP4

Task 4.1

Cellular response of intestinal and liver cells

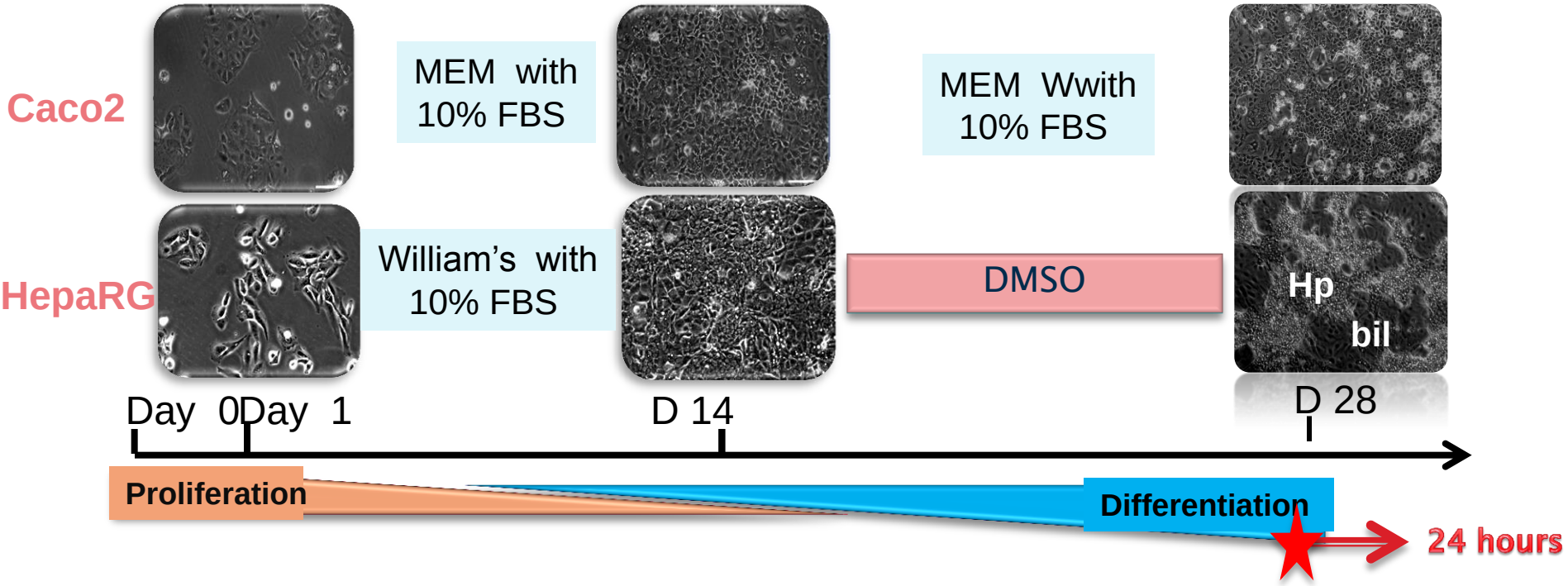
- **Viability**
- **Morphology**
- **Other markers (apoptosis, inflammation, oxidative stress,…)**

Newly developped predictive screening methods like High Content Screening and cell impedancy

WP4

Task 4.1

Cellular response of intestinal and liver cells



Treatment

NM 103 /NM 104

Al

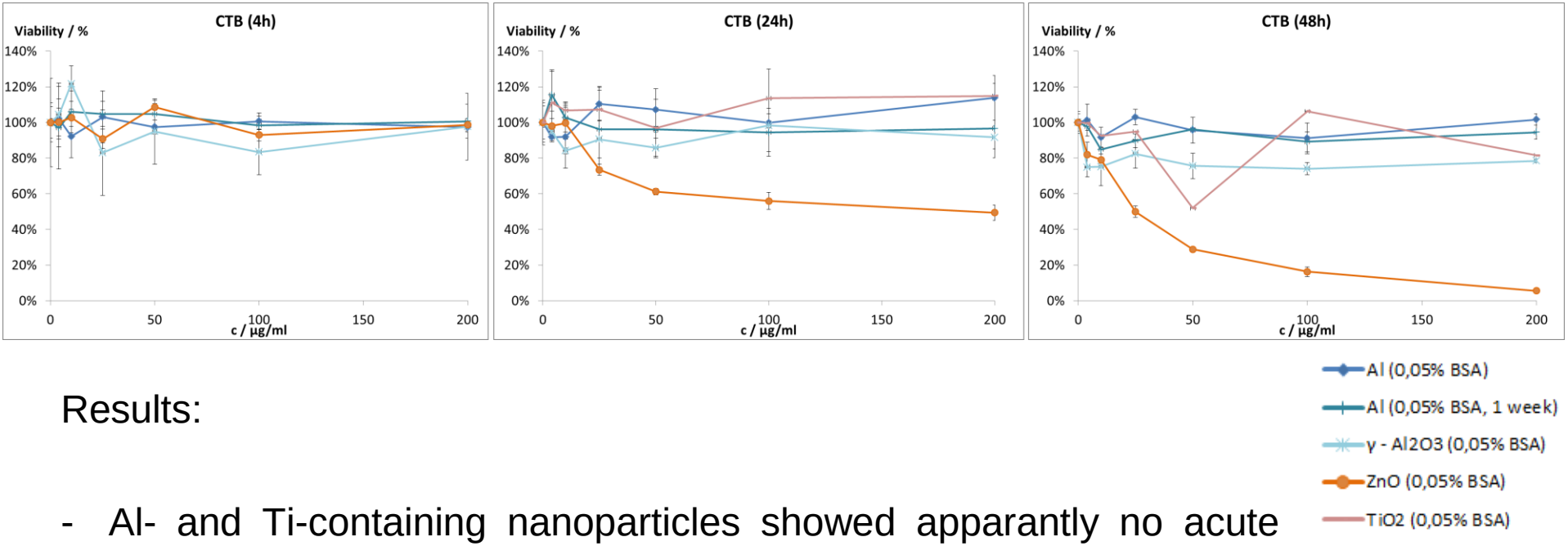
256 → 9 µg/ml

- **Cytotoxicity**: NRU, MTS, casp3 staining (HCS)
- **Genotoxicity** : comet, micronucleus assay, (Y-H2Ax staining (HCS)
- **Cell uptake** :TEM, RAMAN, TOF-SIMS…

WP4

Task 4.1

Cytotoxicity of Nanoparticles on differentiated Caco-2 Cells



Results:

- Al- and Ti-containing nanoparticles showed apparantly no acute cytotoxicity
- Only ZnO particles showed dose- and time-dependent cytotoxicity
- Ionic control substances showed a comparable behaviour

WP4

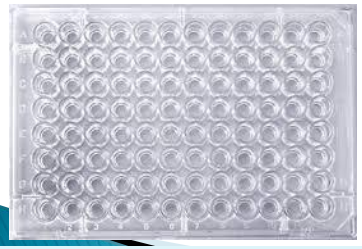
Task 4.1

Toxic effects: First results on TiO2

On differentiated HepaRG

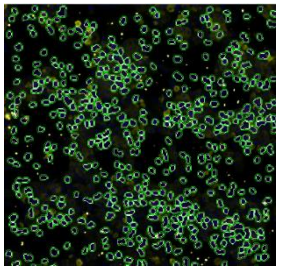
Use of High content analysis to screen several endpoints (at least 3 simultaneously)

Cell count (DAPI), apoptosis (Caspase 3), genotoxicity (H2Ax), inflammation (NFkB), oxidative stress (Nrf2),…



Cells treated for 24h

Fixation and Fluorescent immunostaining



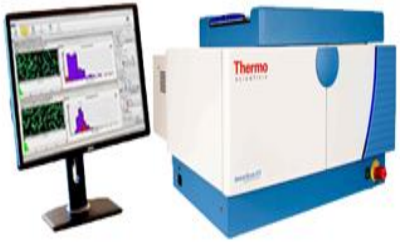


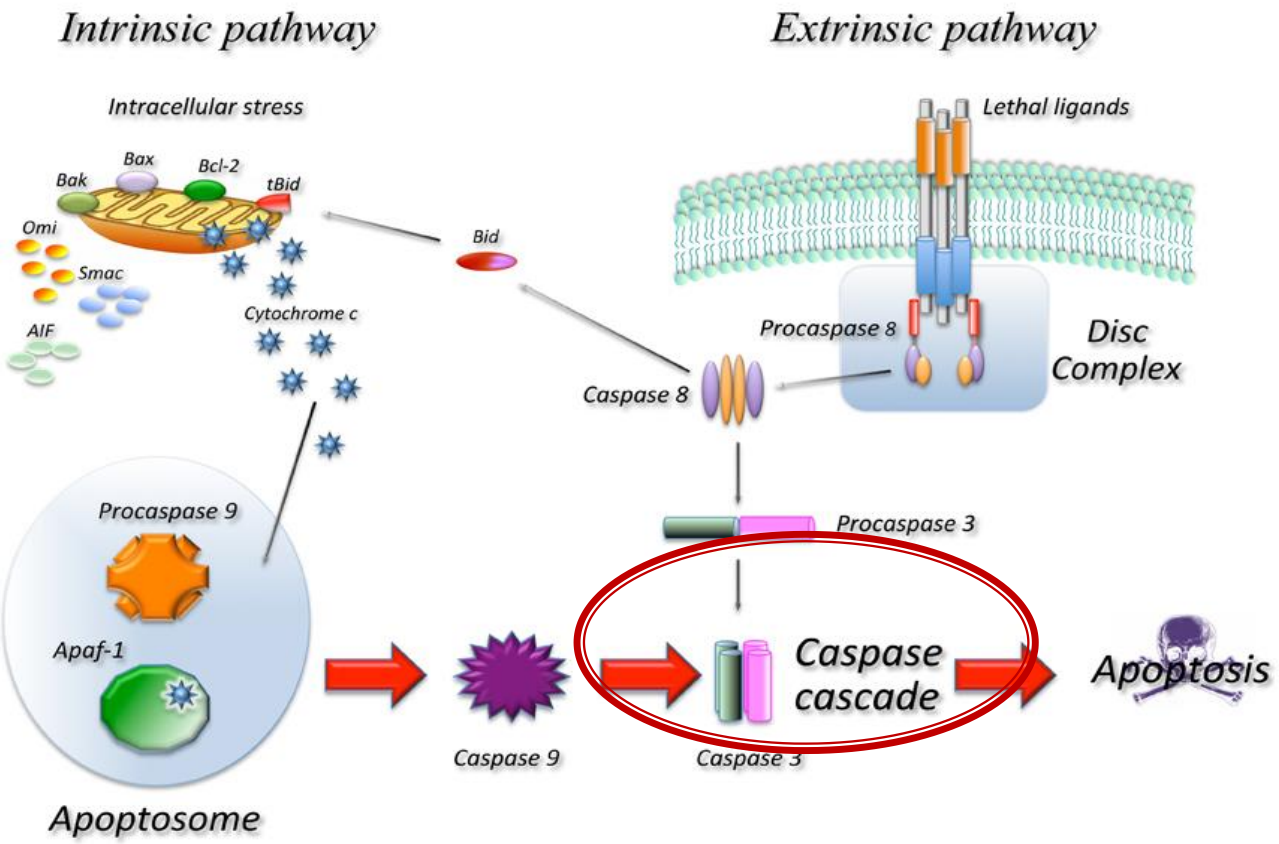
Image analysis

WP4

Task 4.1

Toxic effects: First results on TiO2

Active caspase 3 staining

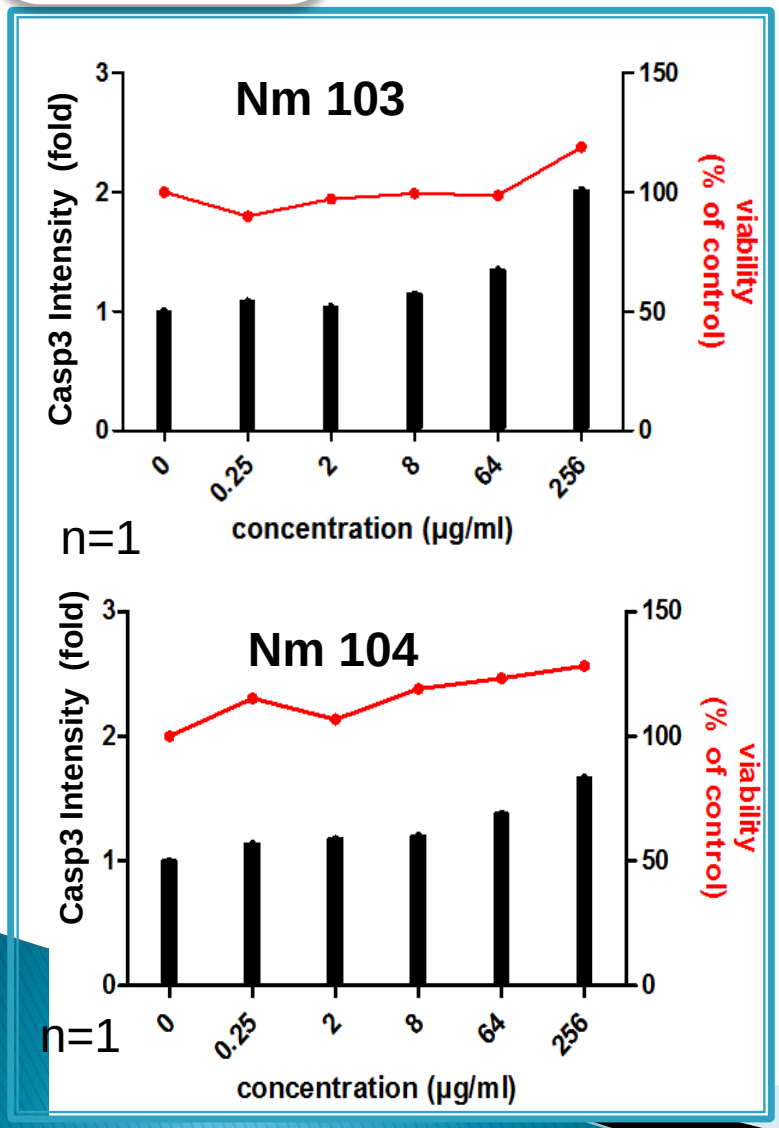


Toxicity → increase of casp3 intensity

WP4

Task 4.1

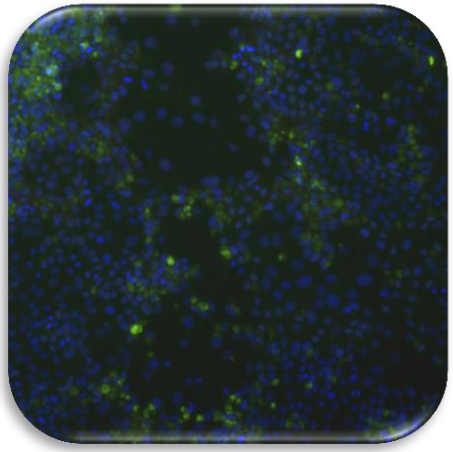
Cellular response of intestinal and liver cells



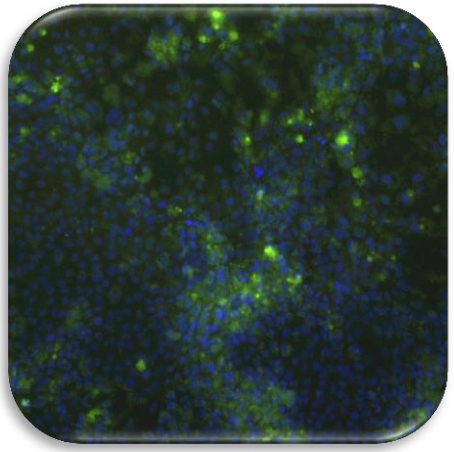
First results on TiO2

Induction of caspase 3 (apoptosis): higher increase induced by NM103

Control



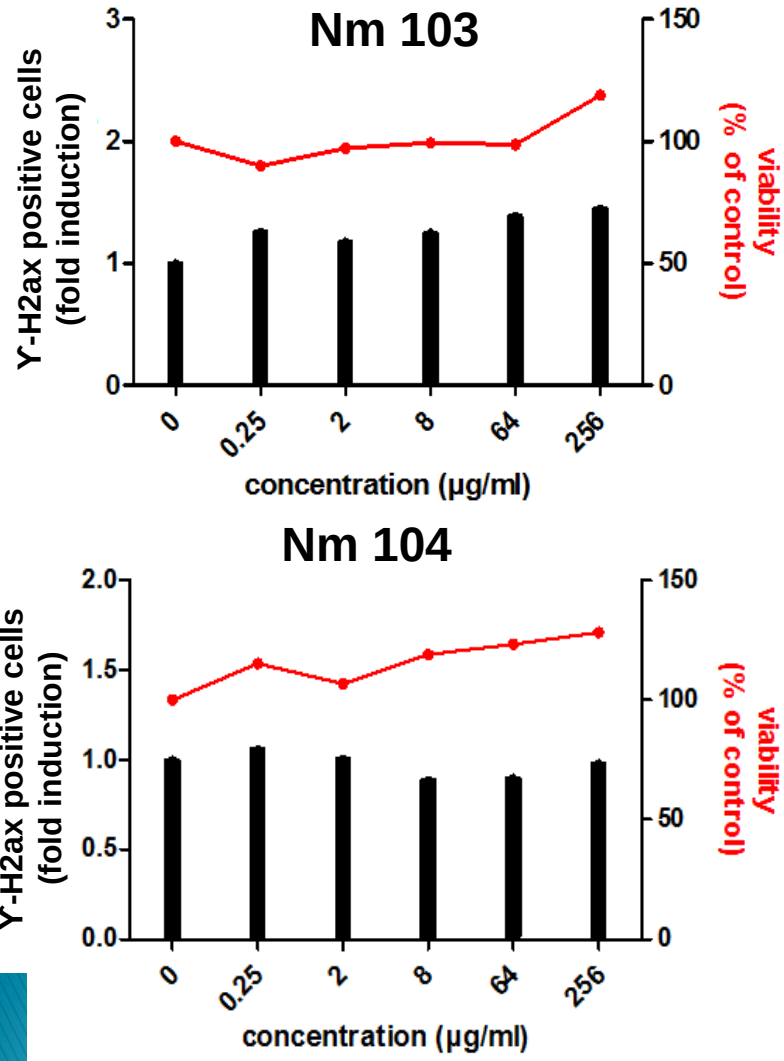
NM103 256 µg/ml



WP4

Task 4.1

Cellular response of intestinal and liver cells



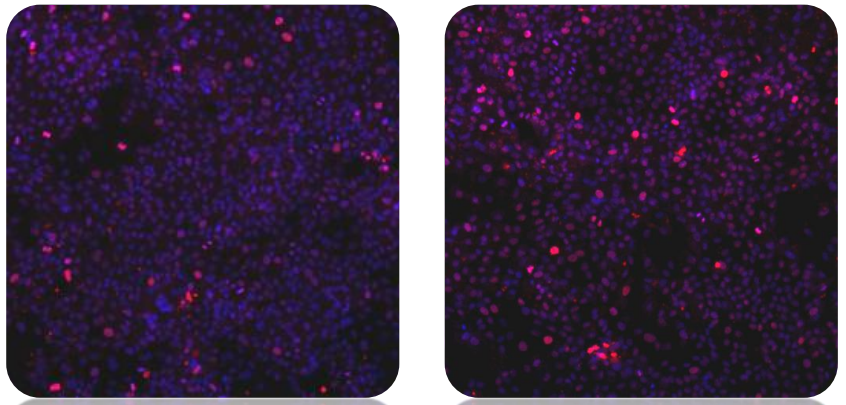
First results on TiO2

H2Ax phosphorylated →Double strand break

No induction of DNA strand breaks with NM103 and NM104

Control

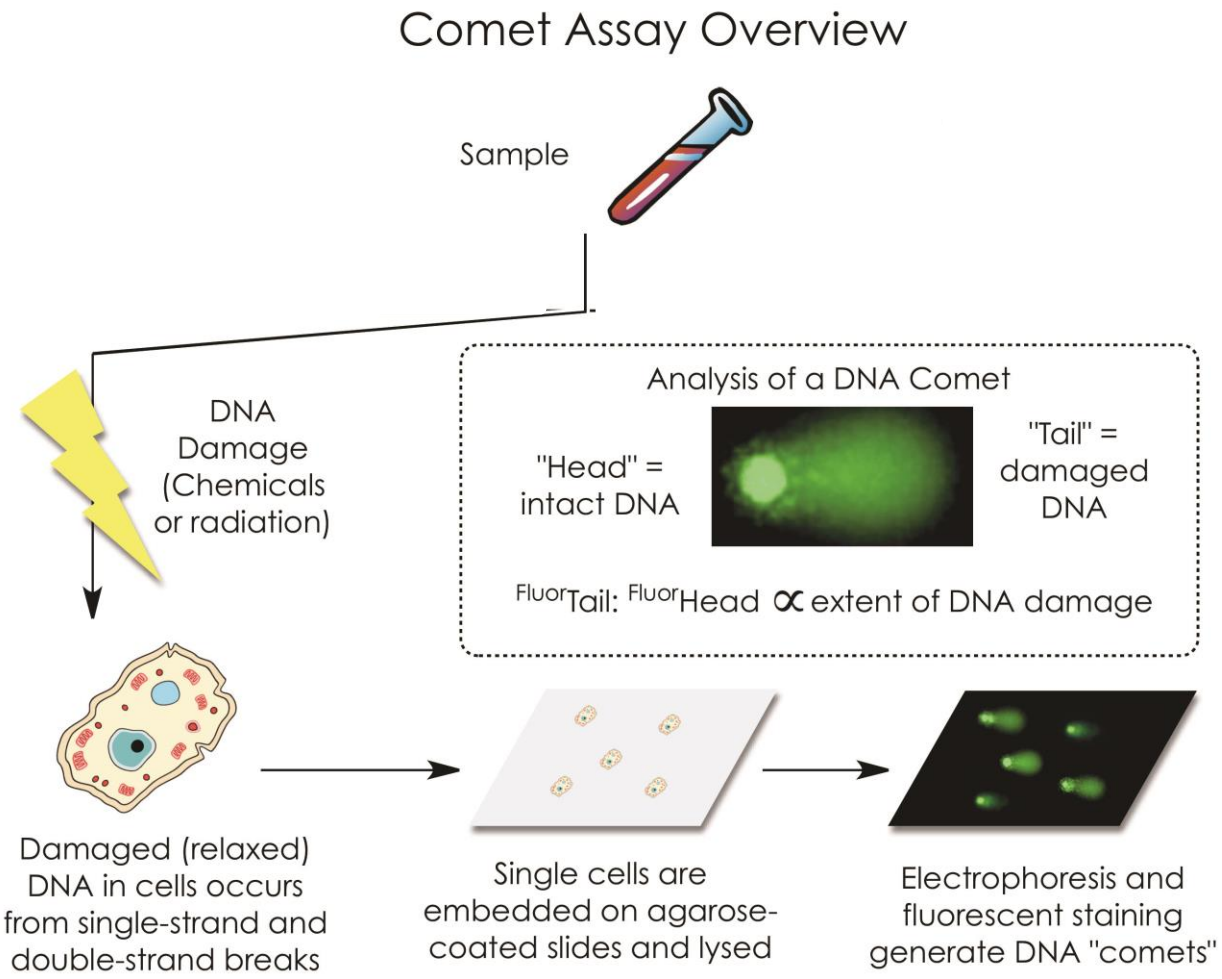
NM103 256 µg/ml



WP4

Task 4.2

Genotoxic effects on intestinal and liver cells

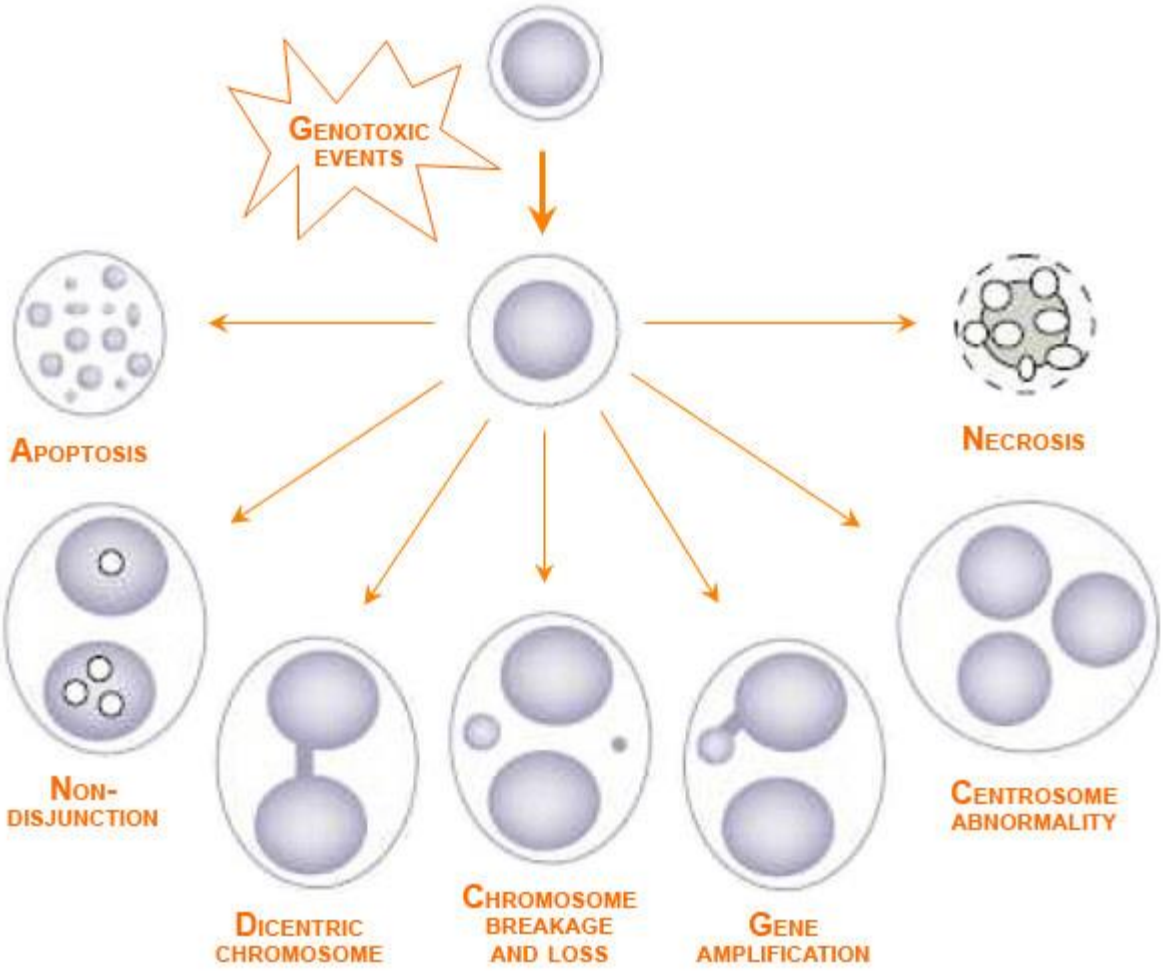


WP4

Task 4.2

Genotoxic effects on intestinal and liver cells

Cytokinesis-blocked micronucleus assay

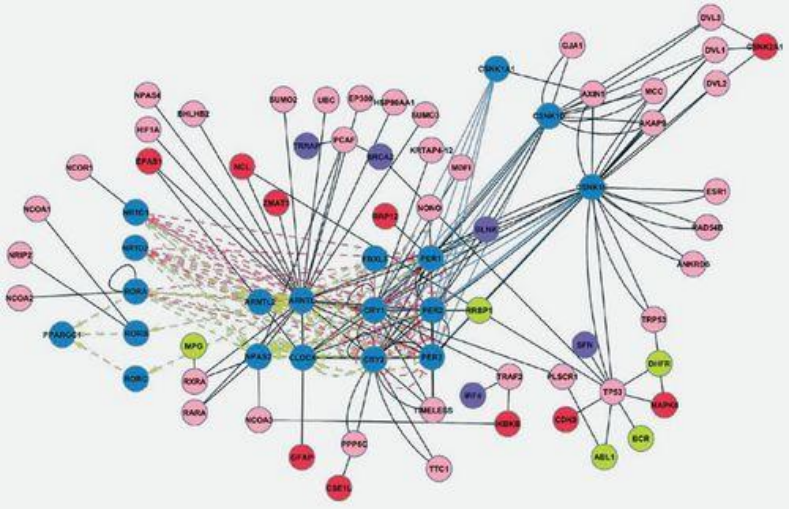


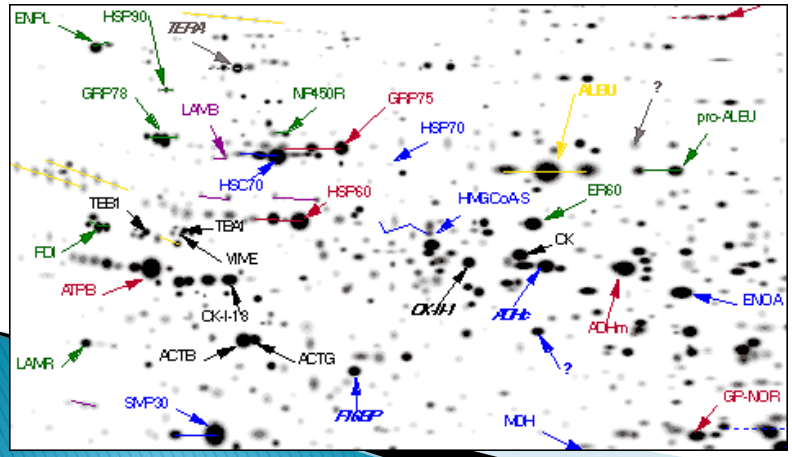
WP4

Task 4.3

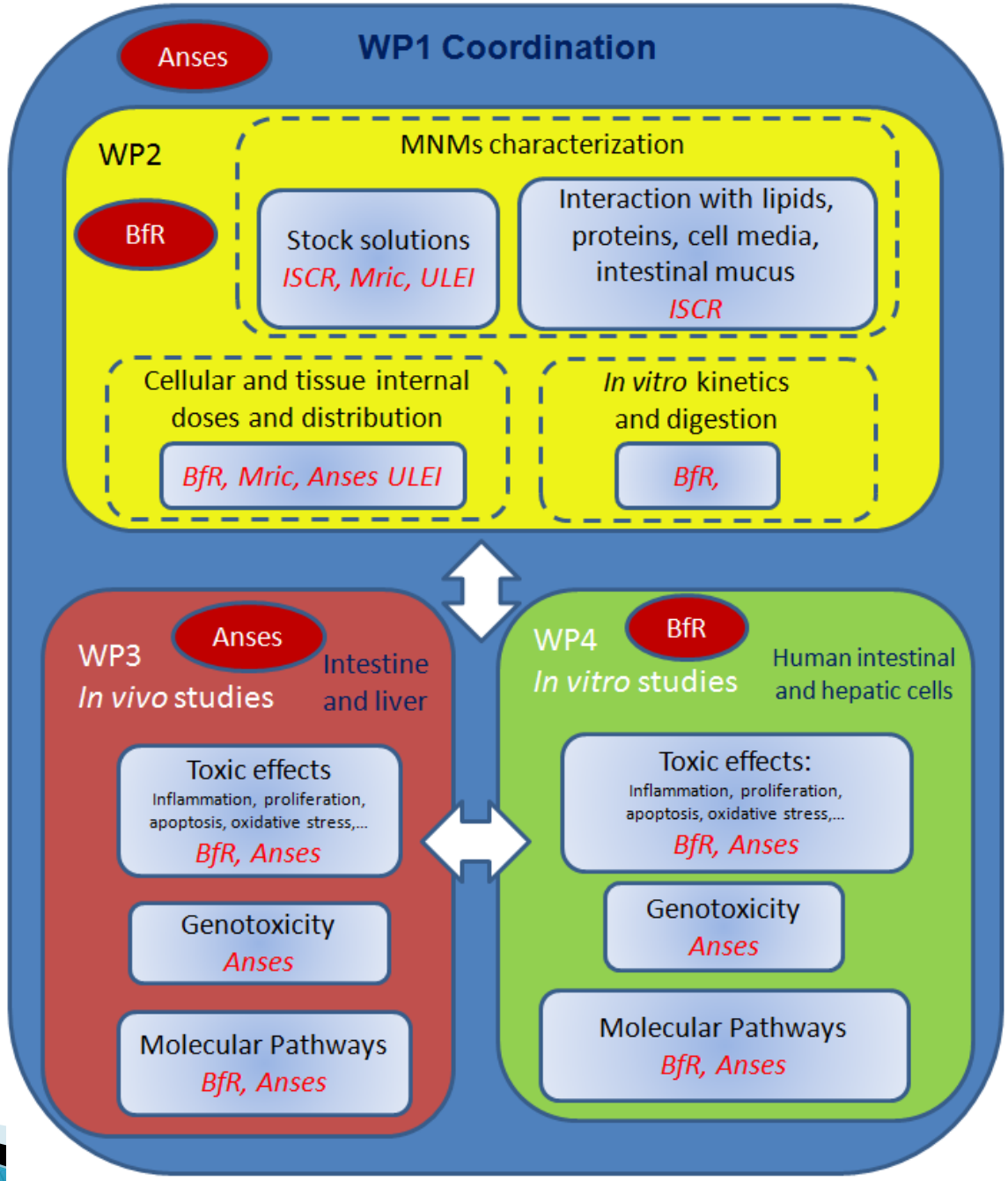
Molecular pathways of MNMs toxicity *in vitro*

Transcriptomics





Proteomics


Anses
WP1 Coordination
WP2
BfR
MNMs characterization
Stock solutions
ISCR, Mric, ULEI
Interaction with lipids, proteins, cell media, intestinal mucus
ISCR
Cellular and tissue internal doses and distribution
BfR, Mric, Anses ULEI
In vitro kinetics and digestion
BfR,
WP3
Anses
In vivo studies
Intestine and liver
Toxic effects
Inflammation, proliferation, apoptosis, oxidative stress,...
BfR, Anses
Genotoxicity
Anses
Molecular Pathways
BfR, Anses
WP4
BfR
In vitro studies
Human intestinal and hepatic cells
Toxic effects:
Inflammation, proliferation, apoptosis, oxidative stress,...
BfR, Anses
Genotoxicity
Anses
Molecular Pathways
BfR, Anses

WP3

Tasks 3.1, 3.2 & 3.3

Genotoxicity of MNMs *in vivo*

Toxic effects of MNMs *in vivo*

Molecular pathways of MNMs toxicity *in vivo*

Gavage, 28 days?

liver, intestine, colon

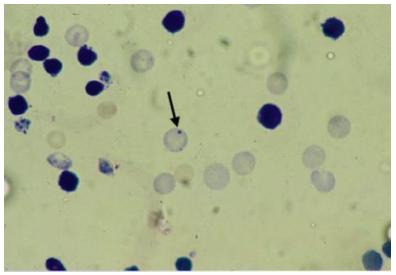
Comet assay

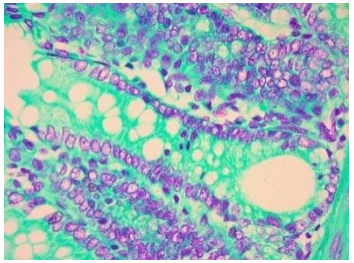
Genotoxicity

Micronucleus assay

Bone marrow (OECD 474)

Colon





liver, intestine, colon, brain

Omics

Uptake
MNM detection

TEM
ToF SIMS
IBM

Histology

Immunostaining for different markers (apoptosis, inflammation,…)

Selected NMs and endpoints according to *in vitro* data

Thank to all the SolNanoTox partners

Especially the **5 students** (4 PhD and 1 master) :

- Thomas Meyer in ULEI
- Benjamin Krause in BfR
- Hoelger Sieg in BfR
- Pégah Jalili in Anses
- Viktoria Ihnatova in ISCR

Choice of MNMs

Table 1: Used materials with analytical data

	Al	γ-Al_2O_3	TiO_2 NM-103 Rutil (hydrophobic)	TiO_2 NM-104 Rutil (hydrophilic)	ZnO	$AlCl_3$ (H_2O)	$ZnCl_2$
Purity	99.9 %	99+ %			99.5 %	99.5 %	99.99 %
APS	18 nm	20 nm	25 nm	25 nm	20 nm	-	-
SSA	40-60 m^2/g	<200 m^2/g	51 m^2/g	56 m^2/g	50 m^2/g	-	-
PM	spherical	spherical	-	-	nearly spherical	-	-